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Inhibition of Prothrombinase by Antithrombin-Heparin at a Macroscopic Surface

Han Speijer, Didier Billy*, George Willems, H. Coenraad Hemker, Theo Lindhout

From the Department of Biochemistry, Cardiovascular Research Institute Maastricht, University of Limburg, Maastricht, The Netherlands

Summary

The antithrombin-dependent inhibition of prothrombinase, assembled at a macroscopic surface, was studied under flow conditions utilizing a tubular flow reactor that consists of a phospholipid-coated glass capillary. Prothrombinase activity was determined from steady-state rates of thrombin production upon perfusion with prothrombin and from factor Va-associated factor Xa activity present in the flow reactor. The prothrombinase density was maintained at a low level (0.03 fmol/cm^2) to assure that the rate of thrombin production reflected the amount of prothrombinase present in the capillary. Perfusion of the flow reactor with antithrombin resulted in an exponential decrease of prothrombinase activity in time. The second order rate constant (8.5 x 10^4 $M^{-1}min^{-1}$) is comparable with the rate of inactivation of free factor Xa. Inhibition was much faster when antithrombin was complexed with heparin. The second order rate constants of inhibition decreased with decreasing heparin chain length: 9.6 x 10^7, 4.5 x 10^7 and 0.39 x 10^7 $M^{-1}min^{-1}$ for unfractionated heparin, low molecular weight heparin and synthetic pentasaccharide heparin, respectively. In the presence of prothrombin (0.2 μM), however, the heparin-dependent rate of inhibition of prothrombinase was about 50-fold lower. The heparin-independent inhibition of prothrombinase by antithrombin (4 μM) in the presence of prothrombin (0.2 μM) was virtually negligible. At a 70-fold higher surface density of prothrombinase (2 fmol/cm^2) prothrombinase activity was much faster inactivated. The rate of thrombin production, however, was not affected. In conclusion, at low prothrombinase densities, prothrombin efficiently protects prothrombinase from inhibition. At high densities, prothrombinase is much less protected but the higher rate of prothrombinase inactivation has no consequences for the thrombin production because of the transport-limited regime.

Introduction

Upon vessel wall damage, blood coagulation is triggered, leading to a number of proteolytic reactions which finally result in the formation of fibrin. One of the central reactions in the cascade of coagulation reactions is the factor Xa-catalyzed activation of prothrombin. The rate of this thrombin-forming reaction is enhanced dramatically by the non-enzymatic cofactor factor Va, calcium ions and phospholipid surfaces (1-3). In vivo, the phospholipid surface is thought to be provided by activated blood platelets or damaged cells present at the site of injury (4-6). In this way propagation of blood coagulation may be restricted to the region of the damaged vessel wall. Termination of the process of thrombin generation is thought to be caused by the thrombin-mediated elimination of the cofactors Va and VIIIa (7) and by the inactivation of the serine proteases factor Xa and thrombin by the plasma inhibitor antithrombin (8, 9). The latter reactions are dramatically enhanced by heparin (10-13), a widely used anticoagulant drug. Yet, inhibition of clot-bound thrombin and factor Xa are hardly affected by heparin (14-16).

A number of studies have demonstrated that phospholipids and factor Va protect factor Xa from inhibition by antithrombin-heparin (17-23). This protection was even more pronounced when the reaction was studied in a tubular flow reactor (24). Recent work from our laboratory (25) has indicated that the catalytic efficiency of prothrombinase in a tubular flow reactor is about two orders of magnitude higher than in a vesicle system. Consequently, inhibition of prothrombinase in a tubular flow reactor could be subject to protection by substrate competition. On the other hand, however, it has also to be expected that because of the extremely high catalytic efficiency of prothrombinase at a macroscopic surface, prothrombin activation can become easily diffusion-controlled (25). Prothrombin concentrations near the catalytic surface then fall to values below the K_m value and protection by prothrombin will be abolished.

In this report, we present the results of a kinetic study on the inhibition of prothrombinase by antithrombin and heparin in a tubular flow reactor. Using different enzyme surface densities, we studied inhibition under conditions where the catalytic activity of prothrombinase sets the pace and under conditions where transport of prothrombin to the catalytic surface limits the reaction rate. As long as thrombin formation was not significantly limited by the transport rate of prothrombin, no inhibition of prothrombinase by plasma concentrations of antithrombin was observed. Only in the presence of heparin significant inhibition of thrombin generation was found.

Experimental Procedures

Materials. S2238, chromogenic substrate for thrombin, was obtained from Chromogenix (Mölndal, Sweden). Bovine serum albumin (fatty acid-free), egg phosphatidylcholine (PC) and brain phosphatidylserine (PS) were from Sigma (St. Louis, USA). Glass capillaries with a length of 127 mm and an internal diameter of 0.65 mm (42 μl volume) were obtained from Brand AG (Wertheim, Germany). The 4th International Standard for Heparin (UFH) was a gift from the National Institute for Biological Standards and Control (Potters Bar, UK). Its potency was stated as 193 I.U. per mg. The low molecular weight heparin (LMWH), enoxaparin (98 anti-Xa IU/mg), was obtained from Rhône-Poulenc Rorer, France. The synthetic pentasaccharide heparin (1140 anti-Xa IU/mg) was from the Choay Institute (Paris, France). The molar concentrations of heparin species with high affinity for antithrombin were determined in each of the preparations by stoichiometric titration of antithrombin (26). The molar amounts of antithrombin high affinity material in 1 g of UFH, LMWH and pentasaccharide were 35 μmol, 29 μmol and 470 μmol, respectively. Bovine

Correspondence to: Dr. TH. Lindhout, Dept. of Biochemistry, University of Limburg, P. O. Box 616, NL-6200 MD Maastricht, The Netherlands – FAX Number: + 31 43 670988

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factor Va, human prothrombin, human factor Xa and human antithrombin were purified and quantified as described (23, 27).

Flow reactor. The preparation of phospholipid-coated capillaries and the flow system were as described previously (25). Briefly, the capillary (0.65 mm internal diameter and 127 mm length) was filled with a suspension of unilamellar phospholipid vesicles composed of 75 mol% egg phosphatidylcholine and 25 mol% brain phosphatidylserine in 50 mM Tris-HCl, 175 mM NaCl, pH 7.9. After 20 min of incubation with the 1 mM phospholipid suspension, the capillary was rinsed with Tris-buffer (50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml bovine serum albumin, pH 7.9) containing 3 mM $CaCl_2$ at a flow rate of 1.2 ml/min for 2 min to remove non-bound phospholipid. The phospholipid-coated capillary was attached to a Hamilton gas-tight syringe. The flow was controlled by a syringe pump (Harvard Apparatus Co., South Natick, Massachusetts, USA). An XYZ translation table (Isel AG, Eiterfelt, Germany) was used to collect samples (typically 30 µl) from the tip of the flow reactor into disposable cuvettes (Sarstedt, Nümbrecht, Germany) containing 20 mM EDTA in Tris-buffer. Thrombin was measured in the cuvette after the addition of the chromogenic substrate S2238. All procedures were performed at 37° C.

Inhibition of prothrombinase activity. The phospholipid-coated capillaries were perfused with a mixture of factor Xa and factor Va (2 nM) for 10 min at 30 µl/min (shear rate 20 s^{-1}) to bind a fixed amount of prothrombinase. The factor Xa concentration in the perfusion mixture was varied to obtain prothrombinase surface densities of about 0.03 and 2 fmol/cm^2. The thrombin production was measured by perfusion of the capillaries with factor Va (2 nM) and prothrombin (200 nM). Samples (30 µl) of the effluent were collected every minute into cuvettes containing 500 µl Tris-buffer containing 20 mM EDTA. After addition of 60 µl chromogenic substrate S2238 (2.5 mM) thrombin was measured as described (25). Inhibition of prothrombinase was studied by perfusion with a mixture containing factor Va (2 nM) and varying amounts of antithrombin or antithrombin-heparin, either in the absence or presence of 200 nM prothrombin.

Determination of factor Xa activity bound to the surface of the phospholipid-coated capillaries. The residual factor Xa activity bound to the phospholipid bilayer was determined at the end of an inhibition experiment. The capillaries were washed for 5 min at 30 µl/min with Tris buffer (50 mM Tris-HCl, 175 mM NaCl, 3 mM $CaCl_2$ and 0.5 mg BSA/ml, pH 7.9) to remove thrombin and were subsequently perfused for 5 min at 30 µl/min with a Tris-EDTA buffer (50 mM Tris, 175 mM NaCl, 5 mM EDTA, 0.5 mg/ml BSA) to remove all bound factor Xa. The factor Xa activity present in the effluent was determined via a bioassay as described (25).

Effect of transport limit on the kinetics of prothrombin activation in the flow reactor. Thrombin generation by prothrombinase bound to macroscopic surfaces easily becomes limited by the transfer of prothrombin from bulk solution to the catalytic surface. Efficient conversion of the prothrombin at the surface results in depletion of the protein in the solution near the surface. The transport of protein to the wall of the tubular flow reactor, J (pmol/min), is proportional to the difference between the protein concentration C_b (pmol/ml) in the bulk solution and C_o (pmol/ml), the protein concentration near the capillary wall (28):

$$J = \Delta (C_b - C_o) \quad [1]$$

with the transport coefficient Δ (cm^3/min) as a function of the geometry of the capillary, the volumetric flow rate, the kinematic viscosity of the fluid and the diffusion coefficient of the protein. For the flow rate of 30 µl/min and the capillary (length 12.7 cm and inner diameter of 0.065 cm) used in this study, the transport coefficient for prothrombin equals 0.0108 cm^3/min (25).

The resulting thrombin production is further determined by the intrinsic conversion kinetics J_{intr} (pmol/min) which depends on the concentration of prothrombin (C_o) at the capillary wall, on the amount of prothrombinase bound to the phospholipid bilayer in the capillary (E), the turnover number of the enzyme (k_{cat}) and the Michaelis constant (K_m):

$$J_{intr} = k_{cat}\, E\, C_o/(K_m + C_o) \quad [2]$$

In a previous study (25) we showed that equations 1-2 allow an adequate description of observed prothrombin conversion kinetics with values k_{cat} = 3600 min^{-1} and K_m = 3 nM.

Results

Inhibition of Prothrombin Activation by Antithrombin

Phospholipid-coated capillaries were perfused with factor Xa (1 pM) and factor Va (2 nM) for 10 min at 30 µl/min. These capillaries were then perfused with prothrombin (0.2 µM) and factor Va (2 nM). The average steady state thrombin concentration at the outlet was 12 ± 0.2 nM (n = 5) and resulted from a steady state rate of thrombin production at the wall of the capillary of about 0.36 pmol/min. This steady state rate of thrombin production is well below the transport limit, $\Delta.C_b$ = 2.2 pmol/min (see Experimental Procedures). We previously demonstrated that under these conditions thrombin production is proportional to the prothrombinase activity (25). The prothrombinase concentration as measured from the amount of factor Xa bound to the phospholipid surface in the tubular flow reactor was 0.09 ± 0.01 fmol (mean ± SD, n = 5). As shown in Fig. 1, perfusion with prothrombin (0.2 µM) and factor Va (2 nM) resulted after 7 min in a stable level of thrombin formation. When perfusion was continued with a mixture containing prothrombin (0.2 µM), factor Va (2 nM) and antithrombin (2 µM), thrombin levels at the outlet of the capillary decreased. The disappearance of the amidolytic activity was due to neutralization of formed thrombin by antithrombin. We emphasize that the absence of thrombin activity at the outlet of the capillary because of inactivation by antithrombin during its residence time of more than 1 min in the capillary does not necessarily mean that the level of active thrombin is reduced at the site of its production. Continuation of the perfusion with prothrombin (0.2 µM) and factor Va (2 nM) resulted in the same thrombin level as before the inhibition step. Perfusion of the capillary at the end of the experiment with EDTA and determination of factor Xa activity in the effluent demonstrated the same molar concentration of factor Xa (0.034 fmol factor Xa/cm^2) as in an untreated capillary (0.037 fmol factor Xa/cm^2). Increasing the antithrombin concentration in the inhibitory perfusion step to 4 µM did not affect the thrombin generating capacity, nor the amount of factor Xa (0.034 fmol factor Xa/cm^2) bound to the phospholipid layer in the capillary. These results thus indicate that prothrombinase inhibition during prothrombin activation by antithrombin is negligible small.

However, when prothrombin was omitted from the perfusion mixture during the perfusion with factor Va and antithrombin, we found that upon perfusion with 2 and 4 µM antithrombin the factor Xa activity in the capillaries decreased from 0.037 fmol factor Xa/cm^2 to 0.020 fmol factor Xa/cm^2 and 0.012 fmol factor Xa/cm^2, respectively: These values paralleled the reduction of the thrombin activity at the outlet of the capillary (Fig. 1). Fig. 2 summarizes the residual prothrombinase activities as assessed from the steady state of thrombin production and factor Xa measurements, after perfusion with varying amounts of antithrombin either in the absence or presence of 0.2 µM prothrombin. It is clearly shown that during prothrombin activation, prothrombinase bound at the phospholipid surface in the flow reactor was virtually insensitive to inhibition by antithrombin. In the absence of prothrombin, prothrombinase inactivation by antithrombin is much faster and increased with the antithrombin concentration.

In order to obtain quantitative data on prothrombinase inhibition in the flow reactor, inhibition by antithrombin in the absence of prothrombin was measured as function of the perfusion time with inhibitor. Capillaries containing prothrombinase at a surface density of 0.022 fmol/cm^2 were perfused with antithrombin (0.5 µM) and factor Va (2 nM) for different time periods. The extent of inhibition of prothrombinase was assessed from the factor Xa activity that was eluted from the capillaries with EDTA. As expected for pseudo-first order kinetics a li-

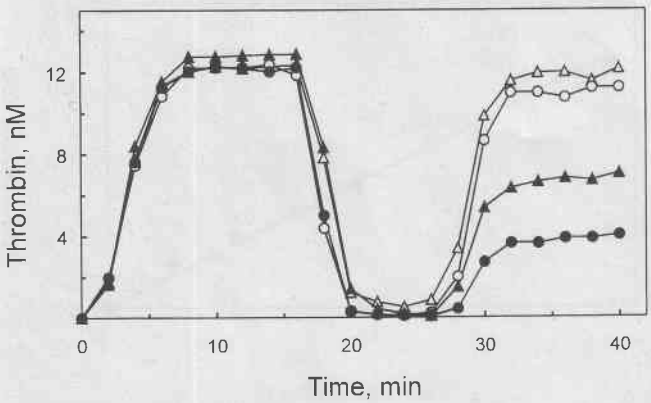

Fig. 1 Inhibition of prothrombinase by antithrombin. Prothrombinase-containing capillaries were prepared by perfusing phospholipid-coated capillaries with 1 pM factor Xa and 2 nM factor Va for 10 min. Prothrombinase densities were 0.037 fmol/cm^2. Then thrombin production was started by perfusion with prothrombin (0.2 μM) and factor Va (2 nM). After 16 min the perfusion mixture was changed to a mixture containing 2 nM factor Va and (△), 2 μM antithrombin and 0.2 μM prothrombin; (○), 4 μM antithrombin and 0.2 μM prothrombin; (▲), 2 μM antithrombin; or (●), 4 μM antithrombin. After 24 min the perfusion was continued with prothrombin (0.2 μM) and factor Va (2 nM). Thrombin concentrations at the outlet are plotted versus the perfusion time. Flow rate was 30 μl/min with a wall shear rate of 20 s^{-1}

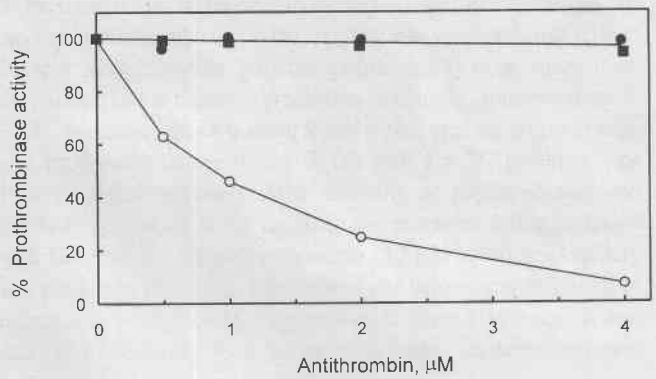

Fig. 2 Effect of antithrombin concentration on prothrombinase inhibition. Prothrombinase-containing capillaries were prepared as described in the legend to Fig. 1. To assess the inhibition of prothrombinase by antithrombin in the absence of prothrombin, the capillaries were first rinsed by a 5-min perfusion with factor Va (2 nM), followed by 8 min perfusion with antithrombin and factor Va (2 nM). Then the capillaries were rinsed again with factor Va (2 nM) and percentage of residual prothrombinase activity (○), was determined as bound factor Xa activity (see Experimental Procedures). Inhibition of prothrombinase by varying antithrombin concentrations was also examined during prothrombin activation. Residual prothrombinase activities (●) were determined by comparing the steady state of thrombin production before and after an 8-min perfusion with antithrombin , prothrombin (0.2 μM) and factor Va (2 nM). Alternatively, inhibition of prothrombinase in these capillaries was also assessed from the amount of factor Xa activity that could be eluted with Tris-EDTA (■). Wall shear rate was 20 s^{-1}

near relationship was found between the logarithm of residual factor Xa activity and the perfusion time (data not shown). A replot of the pseudo first order rate constant of inhibition versus the antithrombin concentration (Fig. 3) yields a second order rate constant of prothrombinase inhibition of 0.85 x 10^5 M^{-1} min^{-1}.

For prothrombinase inactivation during prothrombin activation the extent of inhibition was too small to allow estimation of this rate constant with acceptable accuracy. Only a upper limit can be given. In the presence of 0.2 μM prothrombin the second order rate constant of prothrombinase inhibition is less than 2 x 10^3 M^{-1} min^{-1}.

Inhibition of Prothrombin Activation by Antithrombin/Heparin

In similar experiments as described before for antithrombin alone, we perfused capillaries containing prothrombinase (0.035 fmol/cm^2) with prothrombin (0.2 μM) and factor Va (2 nM) to establish the initial prothrombinase activity of the capillary. Subsequently, antithrombin (0.5 μM) and varying concentrations of heparin were included in the perfusion mixture for 8 min. Residual prothrombinase activity was measured by continuation of the perfusion with prothrombin and factor Va after the inhibition step. At the end of each experiment the capillaries were perfused with EDTA to determine the amount of residual factor Xa activity. We verified, as described for antithrombin alone, that the inhibitory reaction followed pseudo-first order kinetics. Fig. 4 shows the rate constant of inhibition as a function of the heparin concentration. The heparins studied were unfractionated heparin (UFH), the low molecular weight heparin (LMWH) enoxaparin and the synthetic heparin pentasaccharide which contains the antithrombin binding site. Because the antithrombin concentration (0.5 μM) was far above the reported dissociation constant of the heparin-antithrombin complexes (10 nM), we assumed that all antithrombin high affinity heparins were complexed with antithrombin (26, 29). Indeed, the rate constant of the UFH catalyzed reaction increased linearly with the heparin concentration up to 3 μg/ml (100 nM antithrombin high affinity material). The inhibitory activity of the LMWH expressed per mol of antithrombin high affinity material was about half that of UFH. Interestingly, no further increase in the rate constant was seen with pentasaccharide above 1 μg/ml. This amount of pentasaccharide corresponds to a molar concentration of 0.5 μM and above this concentration all antithrombin (0.5 μM) is saturated with the synthetic heparin.

The second order rate constants of inhibition of prothrombinase were calculated from the initial slopes of the plots as shown in Fig. 4 using the molar concentration of heparin as determined by stoichiometric titration with a known amount of antithrombin. We found that the rate constant for the inhibition reaction between prothrombinase and antithrombin-UFH, either in the absence or presence of 0.2 μM prothrombin, was about 30-fold higher than the rate constant of the inhibition reaction with antithrombin-pentasaccharide (Table 1).

Table 1 Second-order rate constants for the reactions of antithrombin or antithrombin-heparin with prothrombin

	Rate Constant ± SE	
	Presence of prothrombin	Absence of prothrombin
	$M^{-1}min^{-1}$ x10^6	
AT	< 0.002	0.085 ± 0.002
AT-H5	0.061 ± 0.001	3.9 ± 0.4
AT-LMWH	0.69 ± 0.01	45 ± 4
AT-UFH	2.1 ± 0.1	96 ± 10

H5, pentasaccharide

AT, antithrombin

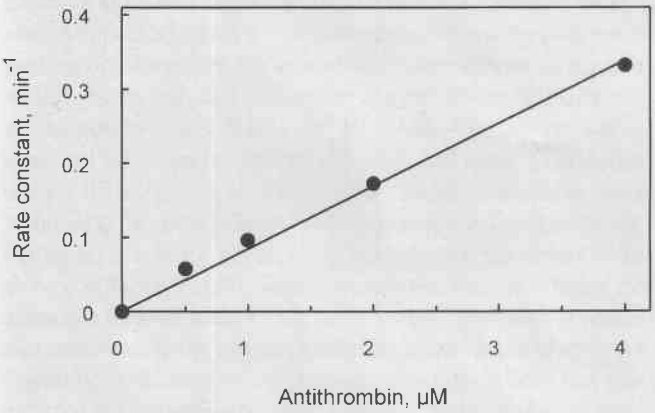

Fig. 3 Rate constant of prothrombinase inhibition as a function of the antithrombin concentration. Prothrombinase-containing capillaries were prepared as described in the legend to Fig. 1. Pseudo-first order rate constants of prothrombinase inhibition by antithrombin in the absence of prothrombin were calculated from the data in Fig. 2. Further details are described in the text

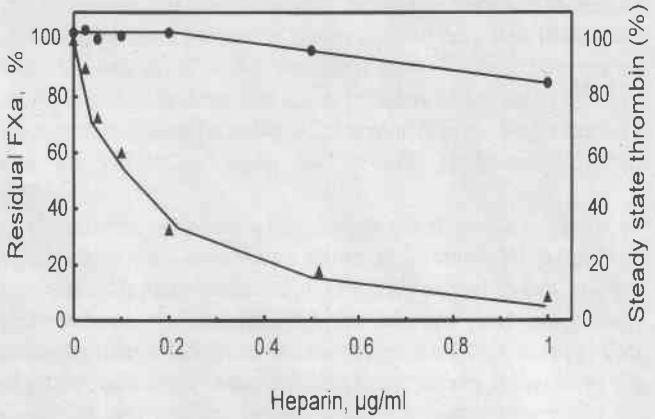

Fig. 5 Inhibition of prothrombinase by antithrombin-UFH under transport-limited conditions of prothrombin activation. Capillaries containing 5.2 fmol prothrombinase were prepared by perfusing phospholipid-coated capillaries with 0.1 nM factor Xa and 2 nM factor Va for 10 min at a wall shear rate of 20 s^{-1}. The capillaries were then perfused for 8 min with 0.5 μM antithrombin and UFH at the indicated concentrations and with a wall shear rate of 20 s^{-1}. The residual prothrombinase activity was assessed as described in the text. Symbols indicate: (●), thrombin production expressed as percentage of the uninhibited steady state thrombin production and (▲), remaining factor Xa activity bound to the capillary as a percentage of factor Xa activity bound to the uninhibited capillaries

Prothrombinase Inhibition During Transport Limited Prothrombin Activation

We next examined the inhibition of prothrombinase by antithrombin/heparin under conditions where thrombin production was limited by the transport of prothrombin to the catalytic surface. To this end, the amount of prothrombinase in the capillary was increased from 0.09 to 5.2 fmol prothrombinase. With this surface density of prothrombinase the intrinsic rate of thrombin production amounts k_{cat}·[prothrombinase] = 18.7 pmol/min, which is about 9-fold higher than the transport limit at a prothrombin concentration of 0.2 μM: J = 2.2 pmol/min (see Experimental Procedures). After assembly of prothrombinase, the thrombin production of the capillary was measured during perfusion with factor Va (2 nM) and prothrombin (0.2 μM). Next, the capillary was perfused with 0.5 μM antithrombin and heparin concentrations as indicated in Fig. 5. Finally, the remaining prothrombinase activity was assessed by determination of the thrombin production during perfusion with prothrombin (0.2 μM) and factor Va (2 nM). At the end of the experiment the amount of non-inhibited prothrombinase in the capillary was determined. Fig. 5 shows that under these conditions the inhibition of prothrombinase in the flow reactor had almost no effect on thrombin generation. It is evident that more than 80% of the prothrombinase had to be inactivated to decrease the rate of thrombin production below the mass transfer rate of prothrombin. This is consistent with our theoretical analysis that the initial amount of prothrombinase was 9-fold the amount at which prothrombin activation becomes transport limited. Half-maximal decrease of prothrombinase was found with 0.14 μg/ml UFH, which equals 5 nM of heparin species with high affinity for antithrombin. It is apparent that in the diffusion-controlled situation, when prothrombinase is only partially saturated with prothrombin, factor Xa is much less protected from inhibition by antithrombin/ heparin. Interestingly, at heparin concentrations higher than 0.2 μg/ml the rate of inhibition of prothrombinase slowed down. It is apparent that sufficient prothrombinase was inactivated to enter a kinetically-controlled situation. That is, the rate of thrombin production became lower than the rate of prothrombin transport to the surface and full protection of prothrombinase by prothrombin is then feasible.

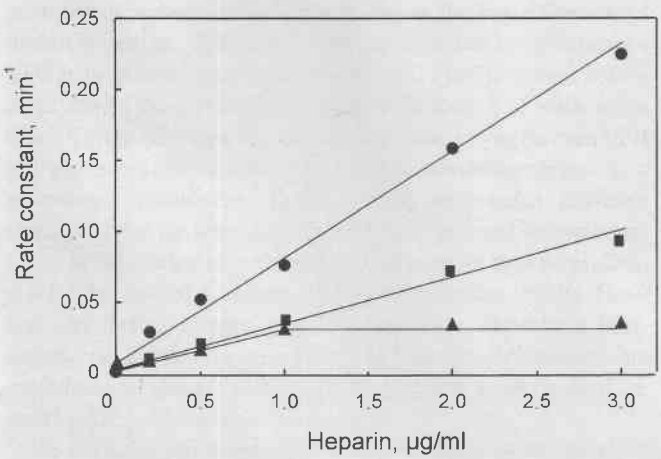

Fig. 4 Rate constant of prothrombinase inhibition as a function of the antithrombin/heparin concentration. Prothrombinase-containing capillaries were prepared as described in the legend to Fig. 1. Pseudo-first order rate constants of prothrombinase inhibition by antithrombin in the presence of varying amounts of UFH (●), LMWH (■) and pentasaccharide (▲) were calculated as described in the text

Discussion

We studied the inhibition by antithrombin-heparin of thrombin production in a tubular flow reactor containing prothrombinase assembled on a macroscopic phospholipid surface. It is demonstrated that under experimental conditions where thrombin production is proportional with the surface density of prothrombinase, antithrombin is a very poor inhibitor of prothrombinase during prothrombin activation. Even at a concentration of antithrombin that exceeds the plasma concentration and a prothrombin concentration that is only one-tenth of the plasma concentration, inhibition of prothrombinase after an 8 min perfusion is almost negligible. The rate constant of inhibition by antithrombin was

estimated to be lower than 2 x 10^3 M^{-1} min^{-1}. We showed that this protection from inhibition is caused to a large extent to the presence of prothrombin, because in the absence of prothrombin we measured a much higher second order rate constant of antithrombin-dependent prothrombinase inhibition (0.85 x 10^5 M^{-1} min^{-1}). This rate constant is somewhat lower than the reported rate constant of factor Xa inhibition of 1.2 x 10^5 M^{-1} min^{-1} (30). Unfortunately, inhibition studies on factor Xa bound to the phospholipid layer in the flow reactor could not be performed because factor Xa readily dissociates from the surface in the absence of factor Va (25). Thus, we could not establish whether the interaction between factor Va and factor Xa at the phospholipid surface also contributes to the protecting effect on factor Xa inactivation. An alternative explanation for the observed protection could be that antithrombin near the surface becomes neutralized by locally high concentrations of thrombin (24). However, at an antithrombin concentration of 0.5 μM the maximal mass transfer equals 5.5 pmol/min (equation 1) which is more than 10-fold higher than the thrombin production of 0.4 pmol/min at the surface. This makes that depletion of antithrombin near the surface is highly unlikely.

Heparin, in the absence of prothrombin, caused a significant acceleration of prothrombinase-antithrombin reaction at the macroscopic surface. Rate constants of inactivation were proportional with the heparin concentration, indicating a second order reaction of prothrombinase with heparin-antithrombin complexes. The second order rate constant for the reaction between prothrombinase and antithrombin-UFH heparin, antithrombin-LMWH and antithrombin-pentasaccharide complexes were 9.6 x 10^7 M^{-1} min^{-1}, 4.5 x 10^7 M^{-1} min^{-1} and 0.39 x 10^7 M^{-1} min^{-1}, respectively. It is of interest to see that the anti-prothrombinase activity of heparin decreased with decreasing heparin chain length. We examined whether this difference between pentasaccharide and unfractionated heparin was the result of an antithrombin-independent effect of the heparins on prothrombinase activity and found that the steady-state rate of thrombin production was not affected by heparin in the absence of antithrombin (data not shown). Earlier studies (31) from our laboratory with factor Xa in the presence of Ca^{2+}, but in the absence of factor Va and phospholipids revealed about the same differences, namely rate constants of 2 x 10^8 M^{-1} min^{-1} and 2 x 10^7 M^{-1} min^{-1} for UFH- and pentasaccharide-antithrombin complexes, respectively.

As demonstrated for the heparin-independent inhibition reaction, prothrombin protects prothrombinase also in the heparin-stimulated inhibition reaction. The second order rate constants of inhibition for UFH and pentasaccharide in the presence of 0.2 μM prothrombin were about 50-fold lower compared to those in the absence of prothrombin (Table 1). The difference in anti-prothrombinase activity between UFH and pentasaccharide reported here for prothrombinase bound to a macroscopic phospholipid surface during prothrombin activation appears to be of the same magnitude (30-fold) as found for prothrombinase in the absence of prothrombin. At present we have no explanation for this marked difference in anti-prothrombinase activity. However, our findings suggest a role for heparin-prothrombinase interactions, in addition to the pentasaccharide-induced antithrombin conformational change, as contributors to heparin acceleration of the antithrombin prothrombinase reaction (30).

We have also performed inhibition studies under conditions where the rate of thrombin production was limited by the supply of prothrombin towards the catalytic surface. In such a situation prothrombinase is then only partially saturated with prothrombin. This, in turn, makes that prothrombinase is more susceptible to inhibition by antithrombin-heparin. Interestingly, in spite of an increased inactivation of prothrombinase, the rate of thrombin production remained unchanged (Fig. 5). The rate of thrombin production will only start to decrease when the activity of prothrombinase at the wall of the flow reactor decreases below the level that causes transport-limited catalysis. Once this situation is reached, however, prothrombin is not longer depleted near the surface and again fully protects prothrombinase from inhibition.

Collectively, our observations suggest that thrombin formation by prothrombinase at a macroscopic surface such as thrombi can hardly be regulated by heparin-antithrombin complexes for two reasons: i) when prothrombinase is saturated with its substrate prothrombin, high concentrations of heparin are needed to cope with the protective effect of prothrombin and ii) when prothrombinase activity is limited by the supply of prothrombin, prothrombinase is readily inactivated by heparin-antithrombin complexes but thrombin formation remains unaffected.

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